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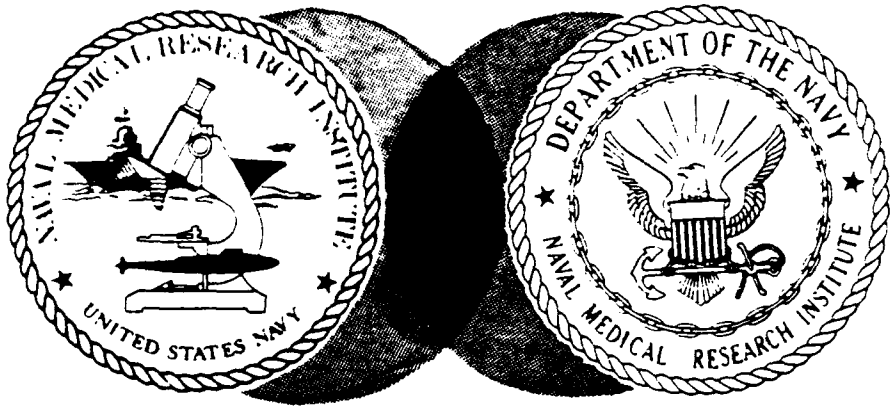

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SPECIFIC CYTOTOXICITY AGAINST AUTOLOGOUS TUMOUR AND PROLIFERATIVE RESPONSES OF HUMAN LYMPHOCYTES GROWN IN INTERLEUKIN 2

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Peripheral blood lymphocytes of cancer patients were sensitized in vitro to autologous tumour cells in mixed lymphocyte-tumour culture (MLTC). Blast cells were isolated on discontinuous Percoll gradients from MLTC which showed significant stimulation of [^{3}H]-thymidine incorporation. Cultured T cells (CTC) were derived from these blasts by growth in conditioned medium containing interleukin-2 (IL-2) and maintained for up to 51 days by repeated feeding with IL-2 and in some cases by addition of irradiated allogeneic blood mononuclear

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cells as "fillers." These cultures showed specific cytotoxic reactivity against autologous tumour and in only a few cases was natural killing (NK) of K562 cells apparent. Restimulation of CTC with tumour was measured in primed lymphocyte tests (PLT). Increased uptake of [^{3}H]-thymidine was found upon stimulation by autologous tumour and allogeneic tumour of the same site and histology but there was no response to non-related tumours or to a panel of allogeneic lymphocytes. No sensitization to autologous HLA D/DR could be detected by restimulation or cytotoxicity against monocytes in the majority of cases. These data suggest that, by careful selection of sensitized blasts from MLTC, it is possible to obtain CTC with both helper (proliferative) and cytotoxic T cells and that such CTC have specific reactivity against tumour cells. These cellular reagents will be useful in defining the antigenicity of human neoplasms and possibly in therapy.

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SPECIFIC CYTOTOXICITY AGAINST AUTOLOGOUS TUMOUR AND PROLIFERATIVE RESPONSES OF HUMAN LYMPHOCYTES GROWN IN INTERLEUKIN 2

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Peripheral blood lymphocytes of cancer patients were sensitized *in vitro* to autologous tumour cells in mixed lymphocyte-tumour culture (MLTC). Blast cells were isolated on discontinuous Percoll gradients from MLTC which showed significant stimulation of [^{3}H]-thymidine incorporation. Cultured T cells (CTC) were derived from these blasts by growth in conditioned medium containing interleukin-2 (IL-2) and maintained for up to 51 days by repeated feeding with IL-2 and in some cases by addition of irradiated allogeneic blood mononuclear cells as "fillers". These cultures showed specific cytotoxic reactivity against autologous tumour and in only a few cases was natural killing (NK) of K562 cells apparent. Restimulation of CTC with tumour was measured in primed lymphocyte tests (PLT). Increased uptake of [^{3}H]-thymidine was found upon stimulation by autologous tumour and allogeneic tumour of the same site and histology but there was no response to non-related tumours or to a panel of allogeneic lymphocytes. No sensitization to autologous HLA D/DR could be detected by restimulation or cytotoxicity against monocytes in the majority of cases. These data suggest that, by careful selection of sensitised blasts from MLTC, it is possible to obtain CTC with both helper (proliferative) and cytotoxic T cells and that such CTC have specific reactivity against tumour cells. These cellular reagents will be useful in defining the antigenicity of human neoplasms and possibly in therapy.

The derivation of cultured T cells (CTC) with specific reactivity against human tumour cells would represent an important advance in studies of the immunobiology of neoplasia. With such reagents, it should be possible to firmly establish the existence, nature and distribution of human tumour-associated antigens and cultured immune T cells might also be valuable for adoptive immunotherapy in cancer patients. In the context of the latter, important studies have been performed by Cheever *et al.* (1981) showing that CTC with specific cytotoxic activity against murine Friend leukaemias, in conjunction with chemotherapy, can be used to eradicate advanced, established tumours. A considerable body of data show that T cells grown *in vitro* in the presence of interleukin-2 (IL-2, previously named T-cell growth factor) retain several important functions, including proliferative and cytotoxic responses to alloantigens (Bonnard *et al.*, 1978; Kurnick *et al.*, 1979; Schendel *et al.*, 1980*a*; Lotze and Rosenberg, 1981). It has been possible to select for cells reactive against specific antigens including autologous EBV-genome-bearing B cells (Tsoukas *et al.*, 1981; Sugamura *et al.*, 1981) by sensitization *in vitro* prior to addition of IL-2 for initiation of the CTC.

Previous studies have established that lymphoid cells from blood, lymph node and tumour-infiltrating lymphocytes from cancer patients, when placed directly in culture in the presence of IL-2, express broad cytolytic activity against both freshly isolated (including autologous) tumour cells and tumour cell line targets (Zarling and Bach, 1979; Vose and Moore, 1981; Kedar *et al.*, 1981; Lotze *et al.*, 1981). Autologous control cells (normal lung or PHA-induced lymphoblasts) were rarely killed. Results with these IL-2-dependent cultured cells may be attributed largely to the presence of polyclonally-activated T cells (Alvarez *et al.*, 1978; Bonnard *et al.*, 1978; Schendel *et al.*, 1980*b*) and also natural killer (NK) cells (Alvarez *et al.*, 1978; Ortaldo *et al.*, 1980). The data contrast with results of direct assays of T-cell-mediated killing where lysis, restricted to autologous tumour targets, was found in approximately 30% of patients (Vose *et al.*, 1977; Vánky *et al.*, 1979; Vose, 1980).

Because a variety of lymphoid cells, including NK cells, grow in response to IL-2, there is a need to develop protocols for obtaining initial selection of T cells with immune reactivity to tumour-associated antigens. One method of particular interest in this respect has been the mixed lymphocyte tumour culture in which 6-day co-cultivation of autologous lymphocytes stimulates DNA synthesis with induction of blasts and the generation of cytotoxicity against autologous tumour target cells (Vánky and Stjernswärd, 1979; Vose *et al.*, 1978; Vánky *et al.*, 1979). Purification of antigen-reactive T-cell blasts from both small resting T cells and NK cells on Percoll gradients prior to addition of CM has improved the specificity of CTC in allogeneic primed lymphocyte tests and cytotoxic assays (Bach *et al.*, 1979; Eckels and Hartzman, 1981; Slease *et al.*, 1981).

In the present report the methods which we have found to be useful for the development of cultured T-cell lines with restricted patterns of cytotoxic and proliferative responses to autologous tumour cells are described. MLTC-blast cultures show preferential cytotoxicity for freshly isolated autologous tumour, but not other autologous cells or, in most cases, the K562 cell line. Proliferative responses assayed in the primed lymphocyte test (PLT) appear to be restricted more by tumour type and histology in that all MLTC-blast CTC were restimulated upon exposure to autologous tumour, allogeneic tumour of the same site but not autologous normal cells or non-related tumours.

MATERIAL AND METHODS

Tumours

The preparation of tumour cells, tumour-infiltrating lymphocytes and macrophages has been described in

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detail elsewhere (Vose, 1981). Briefly, tumour tissues (lung, breast and colon cancer and melanoma) were dispersed in collagenase (Type IV) and the different cell types separated on discontinuous Ficoll-Triosil gradients, by sedimentation through foetal bovine serum (FBS) at unit gravity or adherence to plastic or nylon wool. Separation was started within 3 h of tumour removal and isolated cells cultured overnight to allow regeneration of cell surface structures which had been damaged or removed during separation and which were important for stimulation in MLTC (Vánky and Stjernswärd, 1979). Blood was obtained on the morning of surgery before the administration of any narcotic agent and the mononuclear cells were separated by floatation on Ficoll-Hypaque (LSM, Litton-Bionetics, Kensington, MD). Interface cells were depleted of adherent cells by incubation in plastic culture flasks (Vose *et al.*, 1977). Tumour cells, blood monocytes and lymphocytes were cryopreserved and stored frozen over liquid nitrogen for use as targets and stimulators in tests with CTC.

Mixed lymphocyte-tumour cultures (MLTC)

MLTC assays for measurement of proliferation were performed in 96-well round-bottomed microtest II culture plates (Dynotech Laboratories Inc., No. 1-221-24-1, Alexandria, VA). Constant numbers (1×10^5/well) of responders in RPMI 1640 medium plus 20% heat-inactivated pooled normal human serum were admixed with decreasing numbers of irradiated (3,000 R) autologous or allogeneic tumour cells to give responder: stimulator ratios of between 1:1 and 1:20 (Fig. 1) in 0.2 ml medium. Proliferation was measured by uptake of [^{3}H]-thymidine during a 6 h pulse with 0.5 μCi [^{3}H]-thymidine (sp. act. 6 mCi/mmol) in 20 μl RPMI 1640/well) on day 6 of culture. Previous studies have established that this time of pulse represents the time of maximal [^{3}H]-thymidine uptake. The cells were harvested onto glass fibre discs, then scintillation fluid was added and radioactivity measured in a liquid scintillation counter.

In vitro Sensitization

Bulk cultures in 25 cm^2 culture flasks standing vertically contained 1×10^7 responder lymphocytes with 2×10^6 irradiated tumour cells in 12 ml medium and were incubated at 37°C for 6 days. Tumour-reactive blasts were isolated on discontinuous gradients of Percoll. Gradients consisted of 5 ml-40% 1.5 ml-35% 2.5 ml-31.5% and 2.5 ml-26% Percoll in RPMI and 20% v/v human serum. Cells in 1.5 ml medium were layered onto this gradient and centrifuged (550 *g*, 30 min, room temperature). Blasts were collected from the 31.5% and 35% interfaces and washed twice, then cultures were initiated in conditioned medium as described below. This gradient was particularly useful since it separated T-cell blasts from the more dense, large, granular lymphocytes and small resting T cells which in previous studies have been shown to bond at 40-42.5% and 45-47.5% Percoll respectively.

Conditioned media (CM)

Conditioned media containing IL-2 were produced by PHA stimulation of human lymphocytes in the presence of a B lymphoblastoid cell line as previously described (Bonnard *et al.*, 1980). CM were titrated for activity by their capacity to induce proliferation in IL-2 dependent CTC and used for the maintenance of cell lines at twice the concentration v/v giving maximal stimulation of [^{3}H]-thymidine uptake of such lines.

Cultured T cells (CTC)

Cultures were started in Costar 25 cm^2 culture flasks from MLTI blasts at a concentration of 3×10^5/ml (2 ml) in medium normally containing 10% v/v CM. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and readjusted to 3×10^5/ml every 3-4 days by addition of fresh medium and CM. CTC were used as effectors or responders in functional assays after 8-28 days of growth when cell numbers had increased 100- to 200-fold. Cells were cultured in medium without IL-2 for 24 h prior to testing to reduce spontaneous DNA synthesis for PLT assays and reduce non-specific cytotoxic activity (Ortaldo *et al.*, 1980). In some cases, when more prolonged culturing of CTC was attempted, CTC received irradiated, pooled, allogeneic blood mononuclear cells as feeders at 10-day intervals.

Functional assays

Cytotoxicity of CTC was measured in a 4 h ^{51}Cr release assay as previously described (Vose, 1980). Primed lymphocyte test (PLT) was performed by stimulation of decreasing numbers of CTC (1×10^4/well - 1.25×10^3) with a constant number (5×10^4/well) of irradiated (3,000 R) tumour or control cells. PLT assays were performed in 96-well round-bottomed microtest platest in 0.2 ml RPMI + 20% pooled human serum. Upake of [^{3}H]-thymidine was measured over the last 6 h of a 48 h test as described above.

RESULTS

MLTC

MLTC was performed in 21 cases (8 lung cancers, 9 breast cancers, 2 colon cancers and 2 melanomas). Significant proliferation was recorded in 14 of 21 MLTC and two representative examples are presented (Fig. 1, 2). MLTC were considered positive in the following cases. (1) When mean incorporation in test

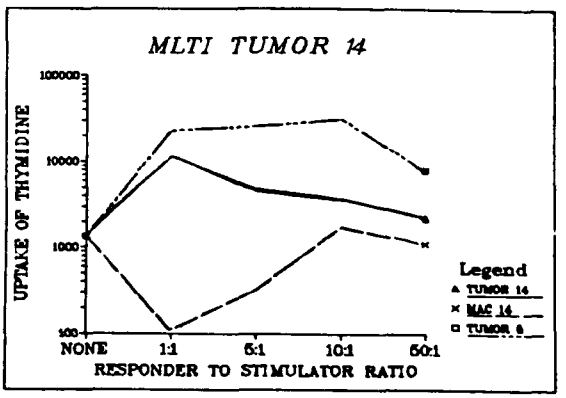

FIGURE 1 – Proliferative response of blood lymphocytes from patient 14 (bronchial adenocarcinoma) to autologous tumour, tumour-derived macrophages (Mac 14) and allogeneic ovarian carcinoma cells (tumor 5) in mixed lymphocyte tumour culture. (proliferative response to PHA: 37,401).

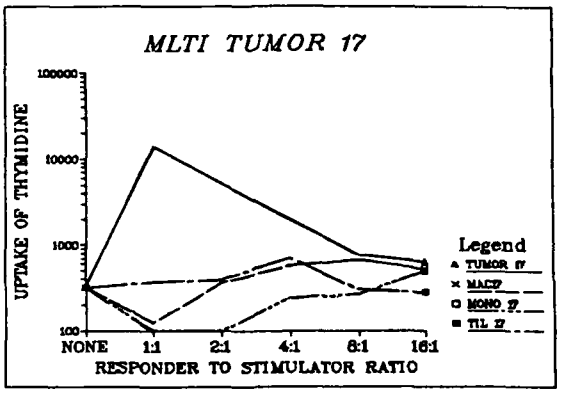

FIGURE 2 – Proliferative response of blood lymphocytes from patient 17 (squamous-cell carcinoma of lung) to autologous stimulator cells. PBL were mixed with decreasing numbers of tumour macrophages Mac 17, monocytes Mon 17 and tumour-infiltrating lymphocytes TIL 17 in RPMI 1640, 20% pooled normal human serum and [^{3}H]-thymidine uptake assessed on day 6 of culture. (Proliferative response to PHA: 23,341 cpm).

cultures with tumour exceeded that of unstimulated cultures by a factor of 3. Since standard deviation of triplicate cultures was less than 8% of the mean, such stimulation values were always significantly different from control (Mann Whitney U-Test). The degree of stimulation depended upon the responder:stimulator ratio, with greatest stimulation occuring at 1:1 in almost all cases and declining with decreasing numbers of tumour cells. In three tumours, the peak of stimulation occured at a responder:stimulator ratio of 5:1. (2) When blasts (more than 10% of surviving cells) were apparent in the cultures. Absence of blasts and lack of stimulation of [^{3}H]-thymidine uptake were the landmark of unstimulated MLTC.

Stimulation was not induced by tumour macrophages, blood monocytes or lymphocytes isolated from the tumour (Fig. 1 and 2). These last two populations were included to compare proliferation in MLTC and autologous MLR (Kuntz *et al.*, 1976). Monocyte/macrophages generally showed inhibition of spontaneous DNA synthesis with high numbers of irradiated cells (1:1-5:1 stimulator:responder). In addition, monocyte-macrophages from cancer patients inhibited both PHA and MLTC stimulation when added at the start of culture (Vose and Moore, 1980), a reaction that was largely attributable to prostaglandin release since it was fully reversible by indomethacin. As cell numbers were reduced, suppression also diminished, but at no point on the dose titration was stimulation of autologous lymphocytes apparent. These data emphasize the importance of adequate removal of monocyte-macrophages from both the responder and stimulator populations, but it is not possible to determine if regognition of macrophage-associated D/DR by T cells occurred against this generally inhibitory background. The exclusion of autologous MLR is clearly of critical importance to the interpretation of MLTC-positive reactions.

Results with MLTC are comparable with those of Vose *et al.* (1977) and Vánky and Stjernswärd (1979). Induction of high levels of proliferation depended on (1) use of viable stimulator preparations; (2) incubation of stimulators overnight following separation; (3) use of stimulators freed of macrophages and lymphocytes which suppress MLTC (above, and Vose and Moore, 1979). These features have been extensively reported elsewhere (Vánky and Stjernswärd, 1979).

MLTC-Blast-CTC

In order to induce cultures with preferential reactivity for autologous tumour, PBL were sensitized *in vitro* in bulk MLTC, then blast cells were isolated and cultured in IL-2-containing CM (MLTC-blast-CTC). Cultures were tested for cytotoxicity and proliferative response to a panel of targets and stimulators between days 8 and 28 of culture. Although CTC could be derived from both MLTC-positive and -negative cultures, the latter showed delayed growth (2/7 did not grow at all) and CTC were not reactive with autologous tumour in PLT (5/5). Cytotoxicity assays showed that these cultures manifested the widespread cytolytic activity of unstimulated CTC from cancer patients (Vose and Moore, 1981). The results with these cultures are not considered further.

Cytotoxicity

MLTC-blast-CTC showed cytotoxicity for autologous tumour in 12/14 cases (Fig. 3, 4, 5). Significant killing was apparent at low effector:target ratios and was dose-dependent. At higher ratios, up to 100% lysis of targets was recorded. In contrast, little reactivity was found against allogeneic tumour even when tumours were matched for histology and site. In Table I, three assays are presented in which CTC were tested against autologous and allogeneic tumour. In all nine cases, autologous tumour was susceptible to CTC kill but no significant allogeneic responses were found. MLTC reactions between tumour and allogeneic lymphocytes also induced kill of the stimulating tumour (*e.g.* allo CTC II) which was again specific. Only two positive reactions against allogeneic tumour were noted in 31 cross-tests. These two tumour targets were also lysed by their autologous MLTC-blast-CTC.

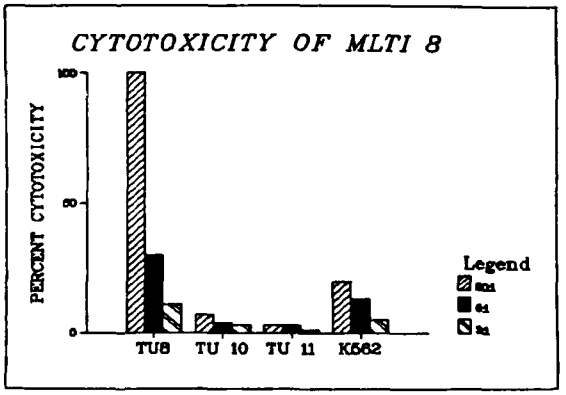

FIGURE 3 – Cytotoxicity of 17-day CTC initiated from MLTC blasts against autologous tumour (Tu 8 head and neck) lung tumour (Tu 11) colon tumour (liver metastasis Tu 10) ovarian tumour (no cytotoxicity data shown) and K562. Cytotoxicity measured in a 4 h ^{51}Cr release assay at effector:target ratios of 20:1, 6:1 and 3:1. Spontaneous releases of ^{51}Cr were: Tu 8 13%, Tu 20 24%, Tu 11 4%, Tu 3 17%, K562 11%.

TABLE I – CYTOTOXICITY OF MLTC-BLAST CTC FOR TUMOUR TARGET CELLS

	% Cytotoxicity[1] against			
	Tumour 7 Ca Gingiva	Tumour 8 squamous CC, lung	Tumour 11 Small-cell Ca, lung	Tumour 14 Adenocarcinoma, lung
CTC 7	28.4*	—	2.0	4.3
CTC 8	7.2	22.0*	2.5	6.1
CTC 11	4.0	2.8	22.1*	—
CTC 14	1.1	3.0	11.2	21.8*
Allo CTC 11 (Healthy donor vs Tu 11)	0	0.8	22.5*	5.2
CTC 25 breast	Tu 25 34.6*	Tu 24 breast 11.1	Monocyte 25 8.7	
CTC 26 breast	Tu 26 15.5*	Tu 25 2.2	Monocyte 26 0	
CTC 15 Squamous CC, lung	Tu 15 38.5*	Tu 16 2.0	Tu 17 1.4	Tu 10 colon 0.8
CTC 16 Squamous CC, lung	0	25.7*	6.2	—
CTC 17 Squamous CC, lung	0.4	0	13.1*	3.8

[1]Effector:Target ratio 5:1. – * $p<0.05$.

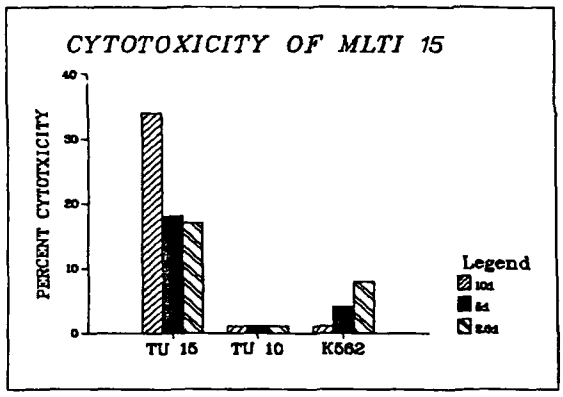

FIGURE 4 – Cytotoxicity of 14-day MLTC-blast-CTC against autologous squamous-cell carcinoma of lung (Tu 15) and allogeneic (colon, Tu 10) tumours and K562.

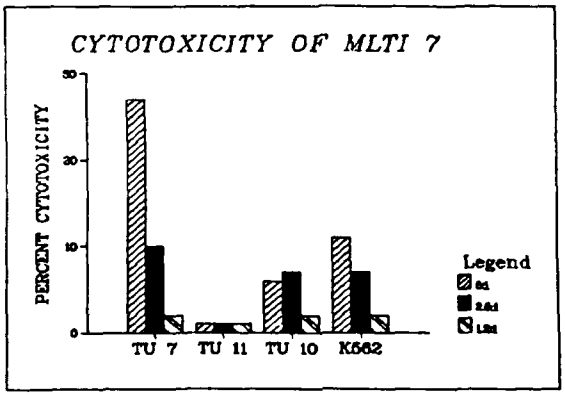

FIGURE 5 – Cytotoxicity of MLTC-blast-CTC 7 (squamous-cell carcinoma of lung) against autologous and allogeneic (Tu 11, small-cell carcinoma lung; Tu 10, colon carcinoma) tumours and K562.

Reactivity against ^{51}Cr-labelled autologous blood monocytes was found in 1/5 assays (patient with adenocarcinoma of lung) and may indicate anti-HLA – D/DR reactivity in this individual. Natural killer activity, as measured by lysis of K562 cell line, was variable but usually low or not detectable at the effector:target ratios used for the demonstration of autologous killing. However some cultured cells did have NK activity (4/14 MLTC-blast-CTC; *e.g.* Fig. 5) and in MLTI-negative cases this often represented the major lytic activity (data not shown). As an additional control, cultures of allogeneic MLTC-blasts (in which lymphocytes from healthy donors were co-cultivated with tumour cells) were initiated. These showed selective reactivity against inducing tumours and levels of killing comparable to those obtained with autologous MLTC-blast-CTC. By using these allogeneic MLTC-blast-CTC it was possible to show that all tumour targets could be lysed by appropriately sensitized CTC. Lack of cytotoxicity against MLTC-negative tumours could not, therefore, be attributed to a resistance to lysis by these target cells.

Proliferative response (PLT)

Restimulation of 13 MLTI-blast-CTC with autologous tumour revealed a significant increase of [^{3}H]-thymidine uptake in all cases (Fig. 6 and 7). Levels of proliferation were low but were comparable to those obtained by addition of fresh IL-2 for this number of cells and culture conditions (Fig. 6) and also to the results of others with CTC (Schendel *et al.*, 1980*a*; Csako *et al.*, 1980). Examples from an extensive series of PLT tests with 11 MLTC-blast-CTC are presented in Table II. Co-cultivation with autologous tumour induced increased incorporation of [^{3}H]-thymidine in all cases with a stimulation index of at least 3.0. Similar increases were induced by allogeneic tumour of the same site and hsitology but tumours from other sites and autologous monocytes were less reactive. MLTC-blast-CTC, in contrast to CTC from peripheral blood

TABLE II – PRIMED LYMPHOCYTE TESTING OF MLTC-BLAST CTC[1]

Culture		[^{3}H]-thymidine incorporation in the presence of					
		Alone	Autologous tumour	Allogeneic tumour same site	Allogeneic tumour different site	Autologous monocyte	Allogeneic lymphocytes
CTC 11	Lung Tumour	500[2]	2207*	—	957*		
14		763	3196*	—	851		
15		921	3089*	2997*	—	839	392
16		631	1841*	—	929	1156	796
17		1114	7302*	3447*	877	892	1022
CTC 24	Breast Tumour	337	1758*	1026*	522	713	651
25		374	1453*	1282*	686	690	642
26		388	1202*	1367*	522	636	
27		332	8431*	1252*	813	646	593
30		402	1595*	931	—	628	803
31		342	3941*	2007*	474	310	607
CTC 74	Colon Tumour	595	2689*	2452*	292	—	—

[1] 1×10^4 responder CTC were co-cultivated with 5×10^4 irradiated 3,000 R stimulator cells for 48 h with incorporation of [^{3}H]-thymidine measured over the last 6 h. Incorporation by stimulators alone was subtracted from the above figures and was always less than 500 counts. – [2] c.p.m.

lymphocytes directly grown in IL-2 without *in vitro* sensitization (Schendel *et al.*, 1980*a*), did not show mixed lymphocyte responsiveness to a panel of allogeneic lymphocytes. For example, MLTC-blast-CTC 15 [squamous-cell carcinoma (SCC) of lung] (Fig. 7) reacted with two other SCC of the lung, but not with a small-cell lung tumour, adenocarcinoma of the lung, ovary or colon, or with AML blasts. Similar results were obtained with MLTC-blast-CTC from nine patients with breast carcinoma in which complete cross-reactivity of restimulation was observed. In two cases of six tested, low reactivity was found against macrophages from the autologous lung tumour. Whether this represents response to macrophages themselves, contaminating tumour cells or absorbed/ingested soluble antigen is under investigation. It would not appear to be attributable to HLA D/DR since there was no significant response to autologous/allogeneic blood monocytes or lymphocytes. The results of all restimulation tests are summarized in Table III. In all PLT assays a slight increase of [^{3}H]-thymidine incorporation was noted in wells containing cells compared with CTC alone. This would appear to be a result of medium conditioning since it also occurred when CTC were mixed with irradiated autologous CTC.

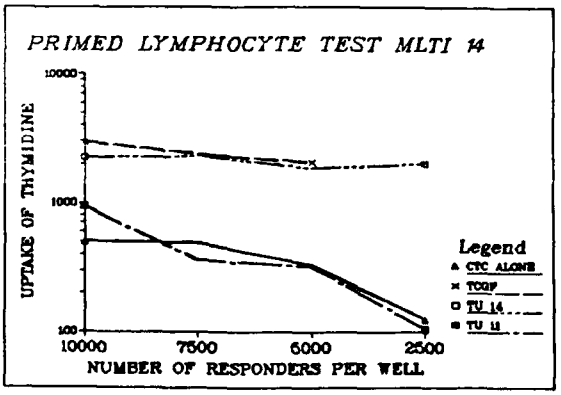

FIGURE 6 – Proliferative response of MLTC-blast-CTC 14 to autologous tumour, allogeneic tumour (Tu 11) and conditioned medium containing IL-2 (TCGF).

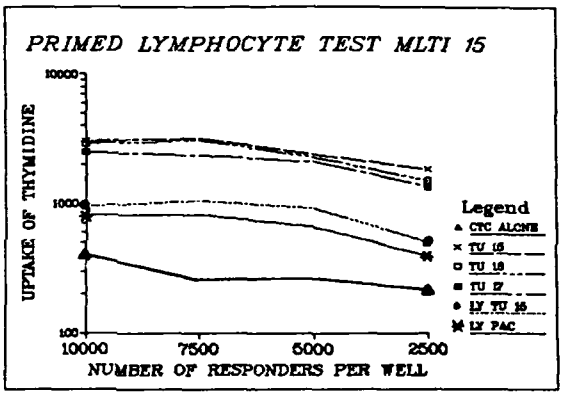

FIGURE 7 – Proliferative response of MLTC-blast-CTC 15 to squamous-cell carcinomas (Tu 15, 16, 17) blood lymphocytes from patient 15 (Ly Tu 15) and a control individual (Ly Pac).

In an attempt to obtain long-term growth of CTC, we employed irradiated, allogeneic, peripheral blood mononuclear cells pooled from two individuals in parallel cultures. These feeders were added at weekly

TABLE III – PRIMED LYMPHOCYTE TESTING OF MLTC-BLAST-CTC – SUMMARY TABLE

Stimulator	No. positive[1] / No. tested
Autologous tumour	13/13
Allogeneic tumour, same site and histology	16/21
Allogeneic tumour, different site	2/14[2]
Allogeneic, same site, different histology	0/ 5
Autologous monocyte	1/ 9
Allogeneic monocyte	1/13
Autologous lymphocyte	1/ 6
Allogeneic lymphocyte	1/18[3]

[1] PLT were considered positive when [^{3}H]-thymidine incorporation of stimulated cultures exceeded that seen in unstimulated cultures by a factor of 3. – [2] One ovarian tumour vs lung (squamous) and one adenocarcinoma lung vs breast. – [3] Unless included as feeders.

intervals to the cultures from initiation from blasts, together with IL-2. By this means some prolongation of culture survival was induced over that of cultures without feeders with CTC regularly reaching 40-50 days. However, cell cultures eventually lost responsiveness to IL-2 and died. Efforts to rescue cultures in crisis using autologous or allogeneic feeders, separation of viable cells on Ficoll gradients or addition of PHA failed. Interestingly, in cultures in which feeders were used PLT responses were apparent against the feeders but not against a panel of five other allogeneic lymphocytes. Cultures were tested for cytotoxic and proliferative activity against autologous tumour at weekly intervals. Both activities were maintained throughout the culture period without changes in levels of reaction. It was observed that, in several cultures approaching crisis, PLT response was apparent where no proliferation was induced by IL-2 alone. This suggests that restimulation of cultures with tumour may be useful in development of continuous lines.

DISCUSSION

NK cells as well as T lymphocytes can grow in conditioned media containing IL-2 (Alvarez *et al.*, 1978; Ortaldo *et al.*, 1980; Timonen *et al.*, 1981). Indeed, we have been able to show by limiting frequency analysis that, upon direct culture of lymphoid cells in the presence of IL-2 and low or undetectable levels of lectins, growth of large granular lymphocytes, which mediate NK (Timonen *et al.*, 1981) often predominates over that of small T cells (Vose and Bonnard, 1981). In addition, the ready induction and growth of polyclonally-activated T cells in at least some CM (Schendel *et al.*, 1980*b*) may result in cultures containing heterogeneous effector populations which obscure the detection of specific immune T cells.

In the present study we have been able to overcome many of these problems by (1) *in vitro* stimulation of lymphocytes with autologous tumour cells and (2) isolation of reactive blasts. The latter step was critical for the demonstration of selective reactivity. Early studies (not reported) showed that MLTC-CTC without blast isolation showed reactivity against autologous tumour but also high NK activity and considerable cytotoxicity against allogeneic targets. The differences in density between MLTC blasts, resting T cells and NK cells offer the basis for this separation (Timonen and Saksela, 1980).

MLTC-blast-CTC, like MLR-stimulated CTC previously studied (Sheehy *et al.*, 1975; Sondel *et al.*, 1976; Bonnard, 1981), showed both proliferative (helper) and cytotoxic T cells which could be maintained for at least 28 days in IL-2-containing supernates. Distinct patterns of reactivity were determined in the two responses measured. Cytotoxicity was strongly restricted to the autologous tumour with activity against allogeneic tumour or autologous monocytes only rarely detected. Such reactivity would be consistent with the expression of individually specific antigens on human tumours or the requirement of histocompatibility between effector and target for T-cell-mediated lysis against common antigenic determinants. This latter possibility was supported by PLT data. Restimulation of MLTC-blast-CTC was found to be confined by tissue site and tumour histology in that complete cross-reactivity was recorded between nine adenocarcinomas of breast and four squamous-cell carcinomas of lung. No PLT response was found using autologous or allogeneic monocytes, lymphocytes or tumours not matched by site and histology. The data of organ-related reactivity are in accord with many studies using a variety of techniques, including cytotoxicity (Hellström *et al.*, 1971; Troye *et al.*, 1977), and leukocyte migration inhibition (Cochran *et al.*, 1973). In the present study the reactions observed could not be attributed to autorecognition of HLA-D/DR antigens but it has yet to be excluded that tissue-specific or differentiation antigens could account for our findings. The number of cross-tests with tumours of different histology and site and with normal tissue preparations is being increased to permit a more definitive identification of the nature and specificitiy of the lymphocyte-stimulating antigens. The data emphasize the value of application of multiple functional assays for the analysis of CTC reactivity. In this respect, the PLT response introduced to the study of leukaemia-associated antigens by Reinsmoen *et al.* (1978) and extended by Csako *et al.* (1980) has been particularly useful. To our knowledge, this present study is the first to document PLT responses in solid tumours. The obvious advantage of the PLT is that stimulation is not restricted by HLA (Csako *et al.*, 1980) and may have some immunodiagnostic role. An intriguing possibility is that cytotoxicity represents a restricted response to common antigenic determinants revealed in the PLT. Alternatively, lymphocyte stimulating and cytotoxic T-cell target antigens may be separate molecules independently expressed, as is the case for alloantigens (Bach *et al.*, 1973).

Current efforts are concentrated on deriving cytotoxic and proliferative T-cell clones with specific anti-tumour responsiveness to approach the many questions raised in this study. A major limitation to the development of cellular reagents for the study of human tumour antigenicity or therapy remains the difficulty of long-term maintenance of CTC. In common with most other workers, we were unable, with the majority of donors, to grow continuous cultures: the most prolonged culture reached only 51 days. Specificity was retained throughout this period. The use of different "feeder" cells for culture maintenance and specific antigen restimulation is currently under investigation.

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